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A fast and simple method for the simultaneous analysis of midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam and 1-hydroxymidazolam glucuronide in human serum, plasma and urine

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- The runtime of the assay for midazolam and its metabolites is only 1.1 minute.
- No deglucuronidation step is needed to quantify 1-hydroxymidazolam glucuronide.
- The validated assay is applicable to quantification in serum, plasma and urine.
- The UHPLC-MS/MS method is suitable for routine analysis and for large studies.

Abstract

For the quantification of the sedative and anesthetic drug midazolam and its main (active) metabolites 1-hydroxymidazolam, 4-hydroxymidazolam and 1-hydroxymidazolam glucuronide in human serum, human EDTA plasma, human heparin plasma and human urine a single accurate method by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has been developed. Protein precipitation as sample preparation, without the need of a time-consuming deglucuronidation step for the quantification of 1-hydroxymidazolam glucuronide, resulted in a simple and rapid assay suitable for clinical practice with a total runtime of only 1.1 minute. The four components and the isotope-labeled internal standards were separated on a C_{18} column and detection was performed with a triple-stage quadrupole mass spectrometer operating in positive ionization mode.
The method was validated based on the “Guidance for Industry Bioanalytical Method Validation” (Food and Drug Administration, FDA) and the “Guideline on bioanalytical method validation” of the European Medicines Agency (EMA). Linearity was proven over the ranges of 5–1500 µg/L for midazolam, 1-hydroxymidazolam and 4-hydroxymidazolam and 25–5000 µg/L for 1-hydroxymidazolam glucuronide, using a sample volume of 100 µL. Matrix comparison indicated that the assay is also applicable to other human matrices like EDTA and heparin plasma and urine. Stability experiments showed good results for the stability of midazolam, 1-hydroxymidazolam and 1-hydroxymidazolam glucuronide in serum, EDTA and heparin plasma and urine stored for 7 days under different conditions. At room temperature, 4-hydroxymidazolam is stable for 7 days in EDTA plasma, but stable for only 3 days in serum and heparin plasma and less than 24h in urine. All four compounds were found to be stable in serum, EDTA plasma, heparin plasma and urine for 7 days after sample preparation and for 3 freeze-thaw cycles. The assay has been applied in therapeutic drug monitoring of midazolam for (pediatric) intensive care patients.

Keywords

UHPLC-MS/MS; Midazolam; 1-Hydroxymidazolam; 4-Hydroxymidazolam; 1-Hydroxymidazolam glucuronide

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Abbreviations: UHPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectrometry; FDA, Food and drug administration; EMA, European Medicines Agency; ICU, intensive care unit; CYP, cytochrome P450; MIDA, midazolam; 1-OH-MIDA, 1-hydroxymidazolam; 4-OH-MIDA, 4-hydroxymidazolam; 1-OH-MG, 1-hydroxymidazolam glucuronide; TDM, therapeutic drug monitoring; SPE, solid phase extraction; SRM, selected-reaction monitoring

The short-acting benzodiazepine midazolam (MIDA) is clinically used as an intravenous sedative drug in critically ill patients admitted to the intensive care unit (ICU) for example to facilitate mechanical ventilation. It is also being administered for its anticonvulsant and muscle-relaxant properties. Furthermore, MIDA can be used as probe substrate for the assessment of cytochrome P450 (CYP) 3A enzyme activity. The metabolic pathway of MIDA is shown in Figure 1. In young healthy volunteers with a normal hepatic and renal function, MIDA has an elimination half-life of 1.5-2.5 hours and is mainly metabolized by CYP3A4 and CYP3A5 to 1-hydroxymidazolam (1-OH-MIDA) and to a lesser extent to 4-hydroxymida-zolam (4-OH-MIDA) [1]. Under the influence of UGT2B4 and UGT2B7, 1-OH-MIDA is conjugated to 1-hydroxymidazolam glucuronide (1-OH-MG), which has a potency of ~10% of MIDA [2, 3, 4]. In critically ill patients, especially with renal failure defined as an eGFR <30 mL/min, accumulation of 1-OH-MG may result in extensive sedative effects [2, 5]. It is our experience that patients with end-stage renal disease may accumulate 1-OH-MG to such an extent, that it may hinder amongst others neurological examination. On the other hand, inhibition of CYP3A4 and CYP3A5 by for example azole antifungal drugs may result in accumulation of the parent drug MIDA. Liver damage resulting in impairment of UGT2B4 and UGT2B7 may result in accumulation of the active metabolite 1-OH-MIDA. Especially in critically ill patients, one or more of these conditions may occur and the importance of analysis both MIDA and its active metabolites is paramount.

To support therapeutic drug monitoring (TDM) and toxicology as for example to help to distinguish between pharmacological effect of (metabolites of) MIDA and neurological damage, a fast and reliable bioanalytical method for the quantification of MIDA, 1-OH-MIDA and 1-OH-MG is necessary. Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the first choice of analytical technique for its good sensitivity and high selectivity. In recent years, several bioanalytical methods have been published for the analysis of MIDA and one or more of its metabolites in human matrices [6, 7, 8, 9, 10]. Some of them described a very sensitive assay for MIDA and 1-OH-MIDA [6, 7] or for MIDA, 1-OH-MIDA and 4-OH-MIDA [8, 9]. However, to achieve the required lower limit of quantification time-consuming sample preparation like solid phase extraction (SPE) or liquid liquid extraction was needed. Together with relatively long runtimes (3-10 min), these assays lack the required speed for urgent samples of (ICU) patients and do not measure the active metabolite 1-OH-MG.

For the quantification of 1-OH-MG in human serum or plasma, only a limited number of publications were identified [7, 8, 10]. Recently the pure substance became commercially available, thereby eliminating the time-consuming enzymatic deglucuronidation step used so far to quantify 1-OH-MG. Additionally, another disadvantage of the deglucuronidation step is the uncertainty of the completeness of deglucuronidation. The deglucuronidation step has been optimized, but a method to prove the absence of any residual 1-OH-MG was not reported [8]. Van Rij et al. [10] were the first reporting a direct analysis of 1-OH-MG, using a “Golden Standard” being a patient’s urine with an extremely high concentration of 1-OH-MG. However, the absence of a pure reference standard does not comply with today’s guidelines for bioanalytical method validation [11, 12]. Moreover, for this assay a large sample volume (1.0 mL), a time-consuming sample preparation and a runtime of 20 min were needed. The assay published by Ahsman et al. [7] incorporated the use of the “Golden Standard” of van Rij et al. [10] and reported a shorter run time (3 min) and a sample volume of 50 µL. Nevertheless, the method contains a time-consuming SPE sample preparation.

serum or plasma and human urine to examine the applicability of the analytical method to different human matrices. Therefore, our goal was to develop and validate a fast and selective LC-MS/MS assay for the simultaneous quantification of MIDA and its main metabolites 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG in human serum, plasma and urine without the need of an extensive sample preparation and a deglucuronidation step for the quantification of 1-OH-MG.

2. Material and methods

2.1. Chemicals and reagents

MIDA, 1-OH-MIDA, 4-OH-MIDA, 1-OH-MG and the stable isotope-labeled internal standards (IS) $^{13}C_6$-midazolam (isotopic enrichment 99.8%; 0.0% unlabeled), $^{13}C_6$-1-hydroxymidazolam (isotopic enrichment 99%; 0.0% unlabeled) and $^{13}C_6$-1-hydroxymidazolam glucuronide (isotopic enrichment 99%; 0.0% unlabeled) were purchased from Alsachim (Illkirch Graffenstaden, France). Because $^{13}C_6$-4-hydroxymidazolam was not available at the time of method development and validation, $^{13}C_6$-1-hydroxymidzolam was being used as the internal standard for both 1-OH-MIDA and 4-OH-MIDA. In the stable isotope labeled internal standards, the six carbon atoms in the chloro-benzene ring of MIDA, 1-OH-MIDA and 1-OH-MG, are replaced by ^{13}C-atoms as displayed in Figure 1.
Methanol (UHPLC-MS quality) was obtained from Biosolve (Valkenswaard, the Netherlands), ammonium formate (99% pure) was purchased from Acros (Geel, Belgium) and formic acid (98-100% pure) was from Merck (Darmstadt, Germany). Ultra-pure water was produced using a Milli-Q system (Millipore Corporation, Billerica, MA, USA).
Drug-free human serum was obtained from Millipore (Temecula, CA, USA), drug-free human EDTA plasma was from Bio-Connect (Huissen, the Netherlands) and blank human heparin plasma and human urine samples were obtained from healthy volunteers according to local hospital standard operating procedures.

2.2. Chromatographic conditions

The UHPLC-MS/MS system consisted of a Thermo Fisher Scientific Vanquish® autosampler (10°C), binary UHPLC pump and column oven coupled to a Thermo Fisher Scientific Quantiva® tandem quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was performed on a Thermo Scientific Accucore C_{18} HPLC column (2.6 µm, 50 x 2.1 mm), maintained at 60 °C. To prevent particles from the injected samples blocking the analytical column, an in-line replaceable frit filter (0.5 µm porosity) was applied. The mobile phase A consisted of ammonium formate 20mM (adjusted to pH 3.5 with formic acid) and mobile phase B was 100% methanol. A gradient was run with a flow rate of 1.0 mL/min. After each injection, the autosampler washes the injection system with methanol:water (80:20, v/v).
The structural similar 1-OH-MIDA and 4-OH-MIDA have the same molecular mass and will have some fragments in common, leading to similar mass transitions. To obtain good selectivity for both 1-OH-MIDA and 4-OH-MIDA, chromatography was optimized to separate the two compounds to prevent interference during positive mode electrospray ionization.

The mass spectrometer operated in the positive mode electrospray ionization using selected-reaction monitoring (SRM). Other mass spectrometer parameter settings were spray voltage 3500 V, sheath gas 50 arbitrary units, auxiliary gas 20 arbitrary units, ion transfer tube temperature 140 °C and vaporizer temperature 350 °C. The resolution of both the first and the second quadrupole was set at 0.7 FWHM, the collision induced dissociation gas pressure was 2 mTorr and the SRM transitions were run with a cycle time of 0.05 sec.

2.4. Standard solution, calibration standard and quality controls

For MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG stock solutions of respectively 1000, 1000, 500 and 1000 mg/L were prepared by accurately weighing the reference compounds and dissolving them in methanol. By diluting the stock solutions with methanol, working solutions were prepared. Independent stock solutions and working solutions were used to prepare calibration and quality control (QC) samples. The total volume of stock or working solution in the matrix never exceeded 5%.

Calibration standards were prepared by spiking the working solutions and stock solutions into blank human serum. The calibration curve consisted of eight calibration standard samples with concentrations ranging from 5 to 1500 µg/L for MIDA, 1-OH-MIDA and 4-OH-MIDA and from 25 to 5000 µg/L for 1-OH-MG.

QC samples were prepared by spiking blank human serum with working solutions and stock solutions reaching concentrations at lower limit of quantification (LLOQ), LOW, MEDIUM and HIGH level. The final concentrations were 5, 25, 500 and 1200 µg/L for MIDA, 1-OH-MIDA and 4-OH-MIDA and 25, 125, 1500 and 4000 µg/L for 1-OH-MG.
To study dilution integrity, human serum was spiked with stock solutions to reach concentrations of 5000 µg/L for MIDA, 1-OH-MIDA and 4-OH-MIDA and 15000 µg/L for 1-OH-MG. For matrix comparison QC samples LLOQ, LOW, MEDIUM and HIGH were prepared by spiking human EDTA plasma, human heparin plasma and human urine with MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG. Calibration and QC samples were stored at -20°C until use and thawed at room temperature before use.

The precipitation reagent, containing the stable isotope-labeled internal standards, was prepared by diluting the internal standard stock solutions of $^{13}C_6$-midazolam, $^{13}C_6$-1-hydroxy-midazolam and $^{13}C_6$-1-hydroxymidazolam glucuronide (1000 mg/L) with methanol until final concentrations of 100 µg/L for $^{13}C_6$-midazolam and $^{13}C_6$-1-hydroxymidazolam and 500 µg/L for $^{13}C_6$-1-hydroxymidazolam glucuronide.

2.5. Sample pretreatment

To obtain a method with a fast turnaround time, sample preparation time needs to be as simple as possible. For this reason, protein precipitation was chosen.
Precipitation reagent (500 µL) was added to 100 µL serum, EDTA or heparin plasma or urine into a 1.5 mL autosampler vial. After vortex-mixing for 1 min, the vials were centrifuged at 9.500g for 5 min. A volume of 1 µL of the supernatant was injected into the UHPLC-MS/MS system.

2.6. Method validation

The developed method has been validated according to the guidelines posted by FDA and EMA for the validation of bioanalytical methods [11, 12]. This validation included selectivity, carry-over, linearity, within-run and between-run accuracy, dilution effect, matrix effect, recovery and stability (benchtop, refrigerator, freeze-thaw and stored in autosampler). Additionally, matrix comparison between human serum and human plasma (EDTA and heparin) and human urine was performed to investigate the application of the assay to these human matrices.

2.6.1. Selectivity

To prove selectivity, six different lots of blank serum, blank EDTA plasma, blank heparin plasma and blank urine were spiked at LLOQ level. These spiked samples and double blank samples are being analyzed and the signal of co-eluting components may not exceed 20% of the peak heights of the analytes at LLOQ and should be less than 5% of the signal of the internal standard.

2.6.2. Carry-over

Carry-over was determined injecting a double blank serum sample after the HLOQ calibrator. The signal of the blank sample needs to be less than 20% of the signal of the analytes at LLOQ and may not exceed 5% of the signal of the internal standards.

2.6.3. Linearity range

Linearity of the calibration curves was tested by analyzing three independent runs on three different days.
The peak height ratio of analyte/IS will be plotted versus the concentration (x) with weighting factor $1/x^2$ to obtain the lowest bias across the calibration ranges. The back-calculated concentrations of all eight calibrators should be within ±15% of the nominal concentrations, except at LLOQ where the calibrator should be within ±20%. At least 75% of the calibrator levels should meet these criteria in each validation run.

2.6.4. Accuracy and precision

Accuracy and within-run and between-run precision were calculated based on the results of four QC levels (LLOQ, LOW, MEDIUM and HIGH), analyzed in fivefold on three different days. At LLOQ level accuracy and precision need to be 80-120% and less than 20% respectively. For LOW, MEDIUM and HIGH QC samples accuracy and precision should be within 85-115% and ±15% respectively.

2.6.5. Dilution effect

OH-MG in human serum were diluted with blank human serum 10-fold and analyzed in fivefold to determine dilution integrity. Accuracy and precision criteria were ±15% of the nominal concentrations.

2.6.6. Matrix effect and recovery of sample preparation

Six lots of blank human serum from individual donors were used for investigating matrix effect and recovery at QC levels LOW, MEDIUM and HIGH.
The matrix factor, expressing the matrix effect, was calculated based on peak heights with and without the presence of matrix for each lot of serum and for each analyte and the internal standards. By dividing the matrix factor of the analytes by the matrix factor of their internal standard, the IS normalized matrix factor was being calculated. The acceptance limit of the CV of the IS normalized matrix factor of the six lots of human serum is 15%.
To ensure efficiency and reproducibility of the sample preparation, recovery experiments were performed. Recovery was calculated by comparing the peak height ratios of the analytes and the internal standards in QC samples in serum with the ratios in extracts of blank serum spiked with the analytes after extraction. The recovery needs to be consistent and reproducible regardless of the concentration of the QC level.

2.6.7. Stability

Stability was tested using LOW, MEDIUM and HIGH QC samples stored at room temperature (20 °C), at refrigerator temperature (5 °C) and in the autosampler (after sample processing, at 10 °C). Also, stability of QC LOW, QC MEDIUM and QC HIGH samples after three freeze-thaw cycles was investigated. Stability experiments have been performed in the matrices human serum, human EDTA plasma, human heparin plasma and human urine. The QC samples were analyzed in fivefold and their concentrations were calculated on freshly prepared calibrators. The analytes were proven to be stable if the concentrations were within 85% and 115% of the initial concentrations.

2.6.8. Matrix comparison

Matrix comparison was performed to investigate whether samples in other human matrices could be calculated on the calibration curves in human serum. QC samples LLOQ, LOW, MEDIUM and HIGH in human plasma (EDTA and heparin) and human urine were analyzed in fivefold. As the concentration of MIDA and its metabolites in urine may exceed the validated concentration ranges, an over-curve sample of 5000 µg/L for MIDA, 1-OH-MIDA and 4-OH-MIDA and 15000 µg/L for 1-OH-MG in human urine was diluted (10-fold) with blank human serum and analyzed in fivefold. The concentrations of the QC samples in human EDTA plasma, human heparin plasma, human urine and the diluted urine sample were calculated on the calibration curve in human serum.
Matrix comparison and dilution of urine with human serum were accepted if accuracy was within 85%-115% (80%-120% for LLOQ) of the nominal concentrations and if precision was less than 15% (less than 20% for LLOQ).

The assay has been implemented in TDM and toxicological casus of MIDA in our hospital. Especially in the management of (pediatric) patients on ICUs and of patients with severe renal failure, fast quantification of MIDA and its metabolites including the glucuronidated metabolite is important.

Case 1

The first case concerns a 72-year-old female patient admitted to the ICU. The patient was still sedated, although MIDA administration (4 mg/h intravenously for 10 hours) has been stopped 3 days before. To confirm that sedation was a toxic effect due to remaining presence of MIDA or its metabolites, blood samples were drawn on 3 consecutive days and the concentrations of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG were measured with the validated method.

Case 2

A 14 days old child admitted to the pediatric intensive care unit because of neonatal convulsions was treated with MIDA 0.9 mg/hour intravenously. For drug monitoring, a blood sample was taken on day 1 after start of infusion. After a dosage adjustment on day 2 to 0.6 mg/h, a second sample was drawn on day 4. On day 6 a third blood sample was taken to determine the concentrations of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG.

2.8. Ethical considerations

Written informed consent was obtained from the patients or their legal representatives to publish the anonymized data.

3. Results and discussions

3.1. Development

Mass spectrometry settings needed optimization as part of method development and to achieve sufficient sensitivity. Mobile phase composition and which column to use have been investigated as well to obtain good chromatographic separation between the compounds.
The structural similar 1-OH-MIDA and 4-OH-MIDA have the same molecular mass and will have some fragments in common, leading to similar mass transitions. To obtain good selectivity for both 1-OH-MIDA and 4-OH-MIDA, chromatography was optimized to separate the two compounds to prevent interference during positive mode electrospray ionization.
For mass spectrometry, often the most sensitive SRM transition will be chosen. For 1-OH-MIDA the highest intensity was found with [M-H_2O], a product ion which normally is a non-specific one. However, being the most sensitive product ion and not being a product ion of 4-OH-MIDA the SRM transition *m/z* 342.1 → 324.0 was chosen for this assay.
The UHPLC-MS/MS system available for this assay, is also being used in daily routine sample analysis. Therefore, the number of combinations regarding mobile phase and analytical column is limited. With method development for the reported assay, ammonium formate 20mM as acidic aqueous mobile phase and methanol as modifier combined with an Accucore C_{18} HPLC column (2.6 µm, 50 x 2.1 mm) resulted in the best analyte responses.

standards are the preferred substances. Because $^{13}C_6$-4-hydroxymidazolam was not available at the time of method development and validation, $^{13}C_6$-1-hydroxymidazolam was being used as the internal standard for both 1-OH-MIDA and 4-OH-MIDA.

3.2. Chromatographic conditions

To achieve an adequate chromatographic separation of 1-OH-mida and 4-OH-MIDA to avoid potential interferences during mass detection, chromatography has been optimized resulting in the gradient shown in Table 1.
With this gradient, the retention times of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG were 0.69, 0.80, 0.56 and 0.48 min respectively.

3.3. Mass spectrometric conditions

For all four compounds good sensitivity was obtained measuring *m/z* $[M+H]^+$ for each precursor ion and the most stable and reproducible product ion. All compound related mass spectrometer settings are presented in Table 2.

The chosen gradient together with the optimized mass spectrometer settings resulted in good chromatographic separation and sufficient sensitivity as shown in the chromatograms in Figure 2. Differences in peak shape and retention times can be observed between panels b, c and d on the one hand and panel e on the other. These were caused by the difference between the two batches of analytical column. The column used in the run for LLOQ and HLOQ was older and of another batch than the one used in the run with this patient sample. To prevent differences in peak performance between various runs, it is advisable if not essential to run a calibration curve in every routine run.

3.4. Method validation

3.4.1. Selectivity

To investigate the selectivity of the developed assay, six individual blank samples of each matrix (human serum samples, human EDTA plasma, human heparin and human urine) were analyzed. The signal of co-eluting components in serum, EDTA plasma, heparin plasma and urine was less than 2.8%, 3.7%, 2.9% and 0.8% respectively of the signal of all four compounds at LLOQ-level and less than 0.03%, 0.02%, 0.02% and 0.2% respectively of the signal of all internal standards. These signals were less than 20% of the signal at LLOQ-level and less than 5% of signal of the internal standard, meaning the method is selective and specific for the analysis of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG and their internal standards in the four investigated matrices.

3.4.2. Carry-over

After injection of the HLOQ calibrator, the signal of a double blank serum sample was 0.3%, 0.5%, 1.6% and 1.2% of the signal at LLOQ level for MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG respectively and 0.00%, 0.02% and 0.09% of the signal for the internal standards.

over was observed.

3.4.3. Linearity range

The calibration curves consisted of eight calibrators with concentrations ranging from 5 to 1500 µg/L for MIDA, 1-OH-MIDA and 4-OH-MIDA and from 25 to 5000 µg/L for 1-OH-MG. The curves were being analyzed on three different days in three independent runs. For all four compounds, the peak height ratios were fitted versus the concentration, using $1/x^2$ as the weighing factor. The averages of the calculated biases of the three calibration runs were -3.9% to 3.0% for MIDA, -3.4% to 2.5% for 1-OH-MIDA, -5.2% to 3.5% for 4-OH-MIDA and -5.1% to 4.1% for 1-OH-MG. The correlation coefficient (R^2) ranged from 0.9994 to 0.9997.
As the calculated biases are low and the correlation coefficients show good linear response over the concentration ranges, the use of peak height ratios to plot the calibration curve was admissible.

3.4.4. Accuracy and precision

The QC samples LLOQ, LOW, MEDIUM and HIGH have been analyzed in fivefold on three different days in three independent runs. The results of these measurements have been used to calculate mean accuracy and within-run and between-run precision which are presented in Table 3. The accuracy ranged from 94.9% to 105.1%, within-run precision was between 0.5% and 4.2% and between-run precision ranged from 0.0% to 1.9%. These results all met the FDA and EMA criteria [11, 12].

3.4.5. Dilution effect

A quality control sample in human serum with over-the curve concentrations for MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG was diluted with blank human serum. This 10-fold dilution resulted in values for accuracy of 95.8% to 106.0% and within-run and between-run precision ranging from 0.7% to 1.4% and from 0.6% to 1.6% respectively for all four compounds.
As the accuracy was within 85% and 115% of the nominal concentrations and the precision was less than 15% for all four compounds, the dilution fulfilled the acceptance criteria [11, 12].

3.4.6. Matrix effect and recovery of sample preparation

The matrix factors of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG and their internal standards, as well as the IS normalized matrix factors and the recoveries at QC levels LOW, MEDIUM and HIGH, are displayed in Table 4.
Using six different lots of human serum, the results of matrix effect and recovery experiments were consistent regardless of the QC concentration. The recoveries were more than 99% with CVs less than 6%, indicating protein precipitation as the sample preparation resulted in high and reproducible extraction efficiencies.

Stability of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG in human serum, human EDTA plasma, human heparin plasma and human urine, was tested under various conditions. The results of these experiments are shown in Tables 5, 6, 7 and 8.
For MIDA, 1-OH-MIDA and 1-OH-MG the accuracy at the investigated conditions was between 94% and 106%, indicating these compounds are stable in human serum, human EDTA plasma, human heparin plasma and human urine for 7 days at room temperature, for 7 days in the refrigerator, for 7 days in the autosampler and after three freeze-thaw cycles.
In human EDTA plasma 4-OH-MIDA was proven to be stable when stored for 7 days at room temperature as the accuracy was more than 86%. As the accuracy of 4-OH-MIDA in human serum and in human heparin plasma after 7 days at room temperature was less than 85%, 4-OH MIDA was found not to be stable in these matrices for 7 days under this condition. The accuracy of 4-OH-MIDA in human serum and in human heparin after 3 days at room temperature being higher than 86%, stability in these two matrices was proven for no more than 3 days. For the analysis of 4-OH-MIDA, serum and heparin plasma samples therefore should preferably be stored in the refrigerator or in the freezer (at -20 °C). Having an accuracy of more than 92% for the stability under the other test conditions, 4-OH-MIDA in human serum, human EDTA plasma and human heparin plasma was proven to be stable for 7 days in the refrigerator, 7 days in the autosampler and after three freeze-thaw cycles.
In human urine, the concentration of 4-OH-MIDA dropped rapidly at room temperature: even after 24h biases were more than 26%. In the refrigerator 4-OH-MIDA in human urine was found not to be stable for 3 days either, as the accuracy of QC LOW and QC MEDIUM was less than 82%. Having biases of less than 8.4% 4-OH-MIDA was proven to be stable in human urine when stored for only 24h in the refrigerator.
Human urine samples therefore need to be stored in the freezer (at -20 °C) until analysis and be shipped on dry ice when transportation is necessary.

3.4.8. Matrix comparison

For matrix comparison QCs in human EDTA plasma, human heparin plasma and human urine were analyzed in fivefold and concentrations of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG were calculated on calibration curves prepared in human serum. The calculated accuracy of all compounds was between 92.8%% and 104.7% for all QCs in human EDTA plasma, with CVs less than 3.3%. For QCs in human heparin plasma, the accuracy and CV were between 90.0% and 110.4% and between 0.1% and 2.5%, respectively. The calculated accuracy of the QCs in human urine ranged from 91.4% to 102.6% with CVs ranging from 0.3% to 5.4%.
As the accuracy of all QCs in the three investigated matrices was between 85% and 115% of the nominal concentrations and the CVs were less than 15%, concentrations of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG in human EDTA plasma, human heparin plasma and human urine can be calculated using a calibration curve prepared in human serum. Over-curve human urine diluted with blank human serum is allowed to be calculated on human serum calibrators as well.

3.5. Clinical application

Case 1

ICU patient on 3 consecutive days, are presented in Figure 3.
As can be expected after stopping the MIDA administration 3 days before the first blood draw, the MIDA concentration was lower than the validated LLOQ (5 µg/L). The concentration of 1-OH-MG was 1700 µg/L on the first day of drug monitoring. As 1-OH-MG is ~10% as potent as MIDA and sedation is achieved at MIDA concentrations starting at 150 µg/L, the sedated condition of the patient can be explained by this 1-OH-MG concentration [2, 4, 5].

Case 2

On three different days the serum concentrations of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG of a 14 days old child treated with MIDA for neonatal convulsions were measured. In Figure 4 the results of these analyses are presented.

Both cases show the importance of the direct measuring of 1-OH-MG as well as the validated concentration ranges of the four compounds MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG being clinically relevant.

4. Conclusion

A simple, rapid and accurate UHPLC-MS/MS method for the quantification of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG using 100 µL of human serum has been developed and validated. Especially the lack of a time-consuming deglucuronidation step for the quantification of 1-OH-MG makes it a very fast assay. The structural similar 1-OH-MIDA and 4-OH-MIDA have been chromatographically separated to prevent interference. With matrix comparison the use of human EDTA and heparin plasma and human urine has also been approved. Because of proven instability of 4-OH-MIDA especially in human urine, samples need to be frozen until analysis or when being transported.
Having a runtime of 1.1 min, the method is appropriate for the analysis of urgent samples for TDM as well as of large numbers of samples in pharmacokinetic studies. The applicability of this method has been proved in representative clinical cases.

Declaration of interests

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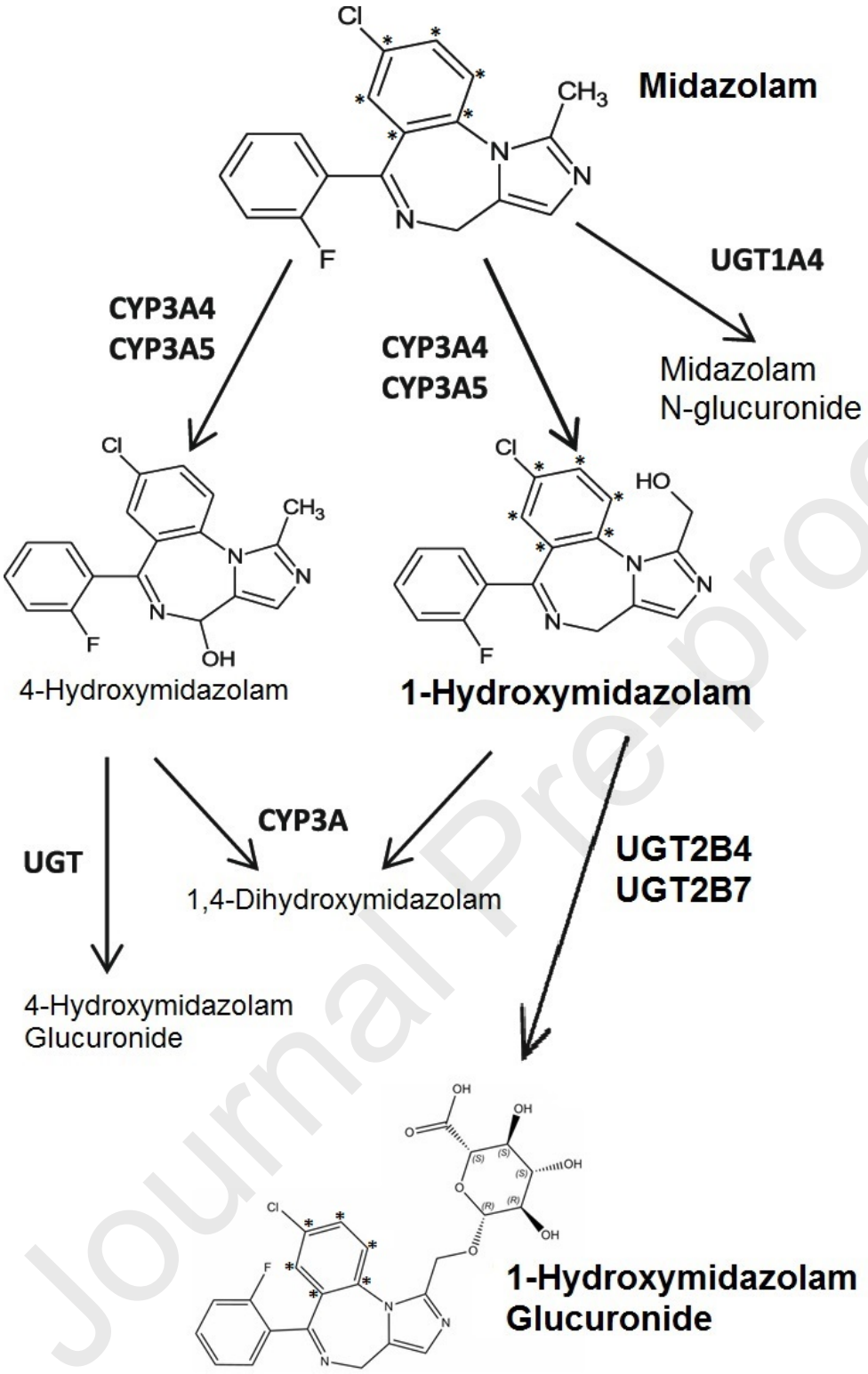
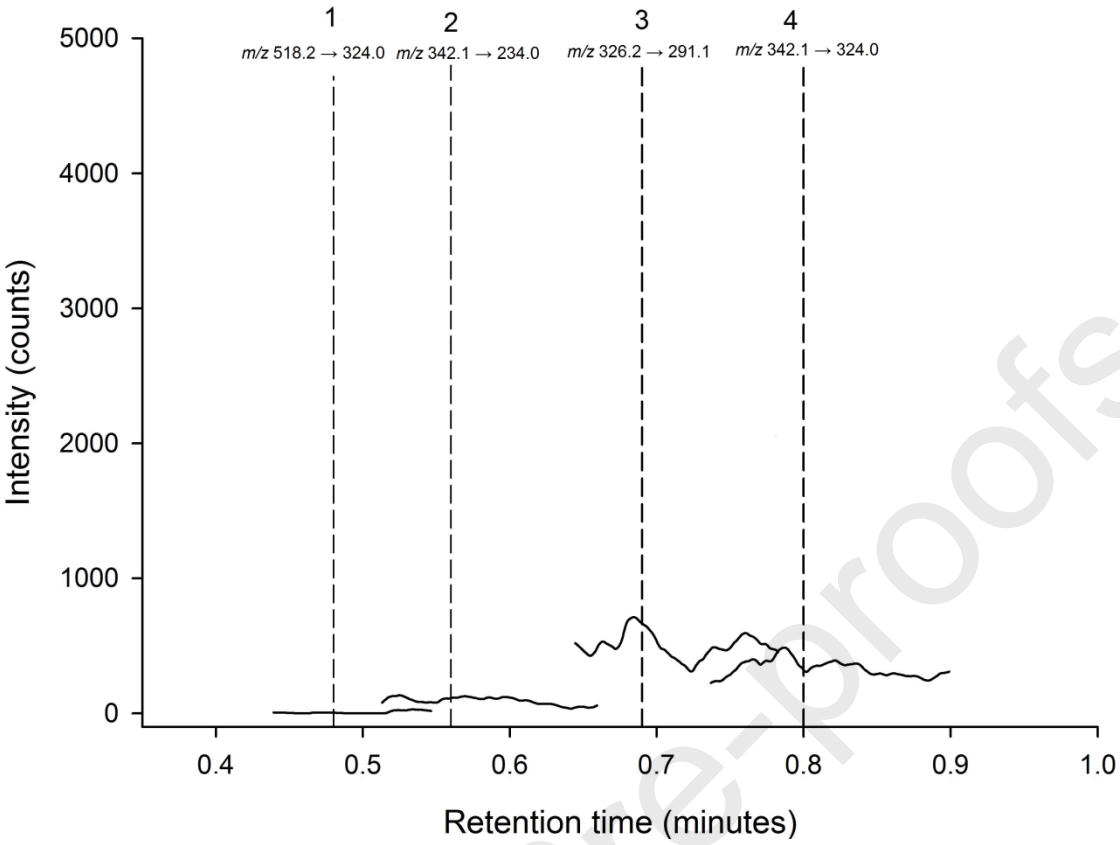
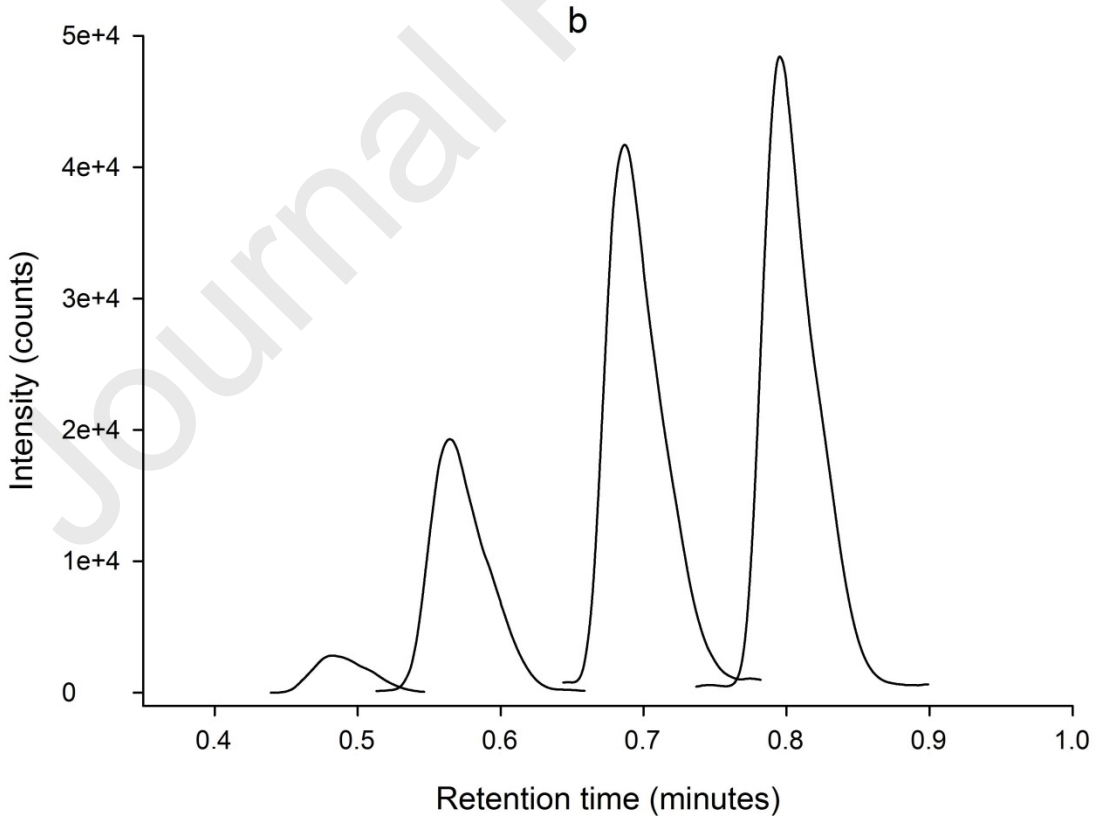
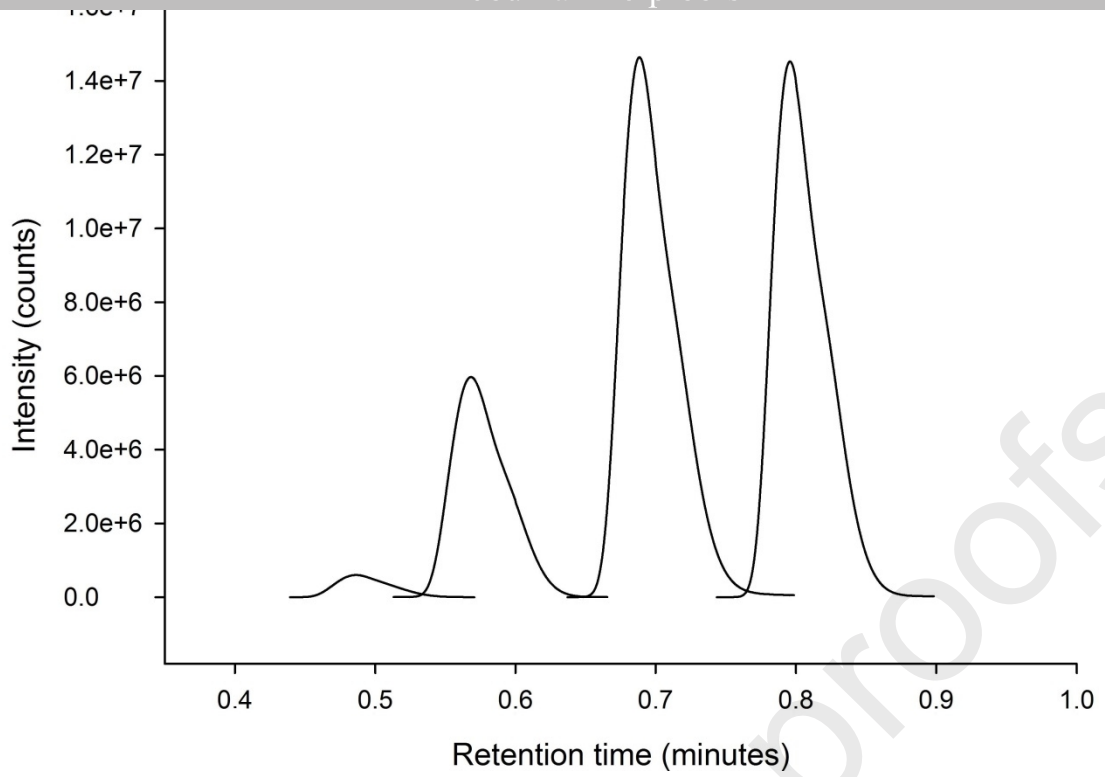
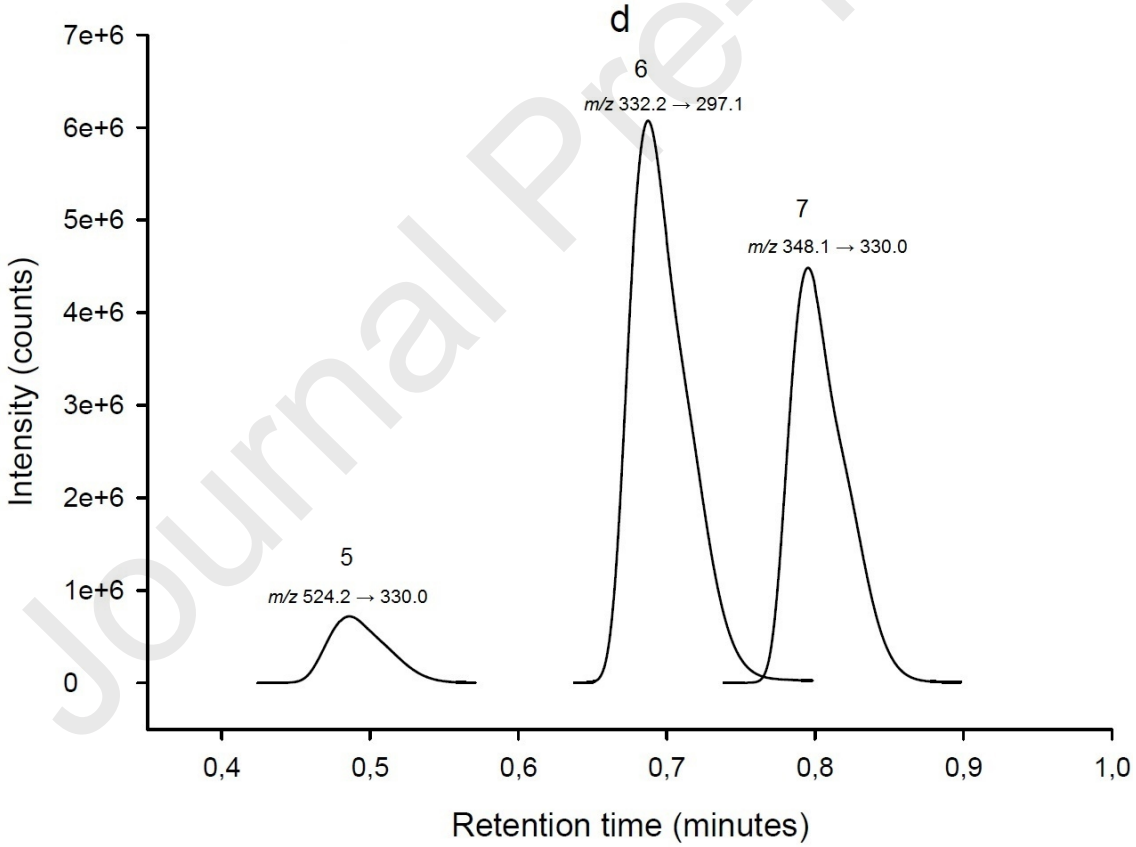


Figure 1: Metabolic pathway of midazolam. The compounds in bold represent midazolam and its active metabolites. The positions of the ^{13}C labels are indicated by an asterisk (*).









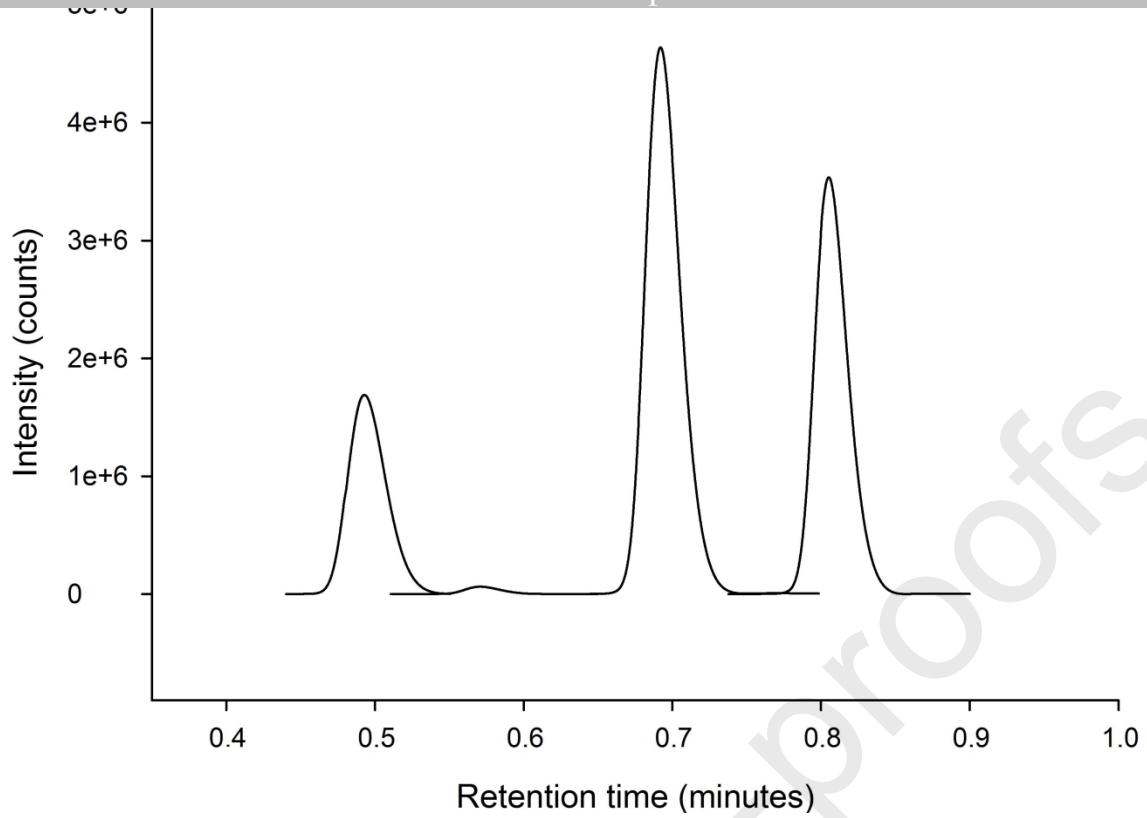


Figure 2: Chromatograms of blank human serum (a), the LLOQ in human serum (b) the HLOQ in human serum (c) for the analytes 1-OH-MG (1), 4-OH-MIDA (2), MIDA (3) and 1-OH-MIDA (4) and the internal standards (d) $^{13}C_6$-1-hydroxymidazolam glucuronide (5), $^{13}C_6$-midazolam (6) and $^{13}C_6$-1-hydroxymidazolam (7). Panel e shows the chromatogram of a patient's serum sample containing MIDA (330 µg/L), 1-OH-MIDA (380 µg/L), 4-OH-MIDA (15 µg/L) and 1-OH-MG (4100 µg/L).

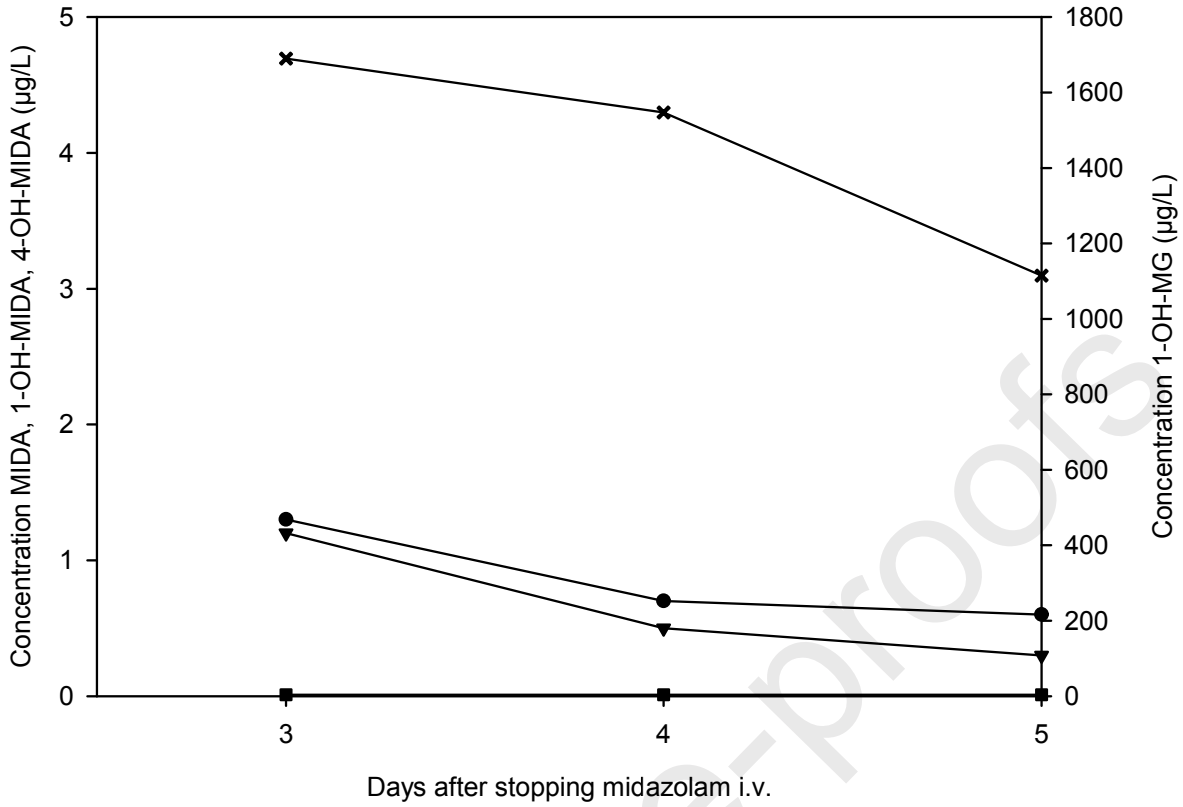


Figure 3: Concentrations of MIDA (●), 1-OH-MIDA (▼), 4-OH-MIDA (■) and 1-OH-MG (**x**) in sedated ICU patient after stopping midazolam administration (4 mg/h intravenously for 10h) three days prior to measurements

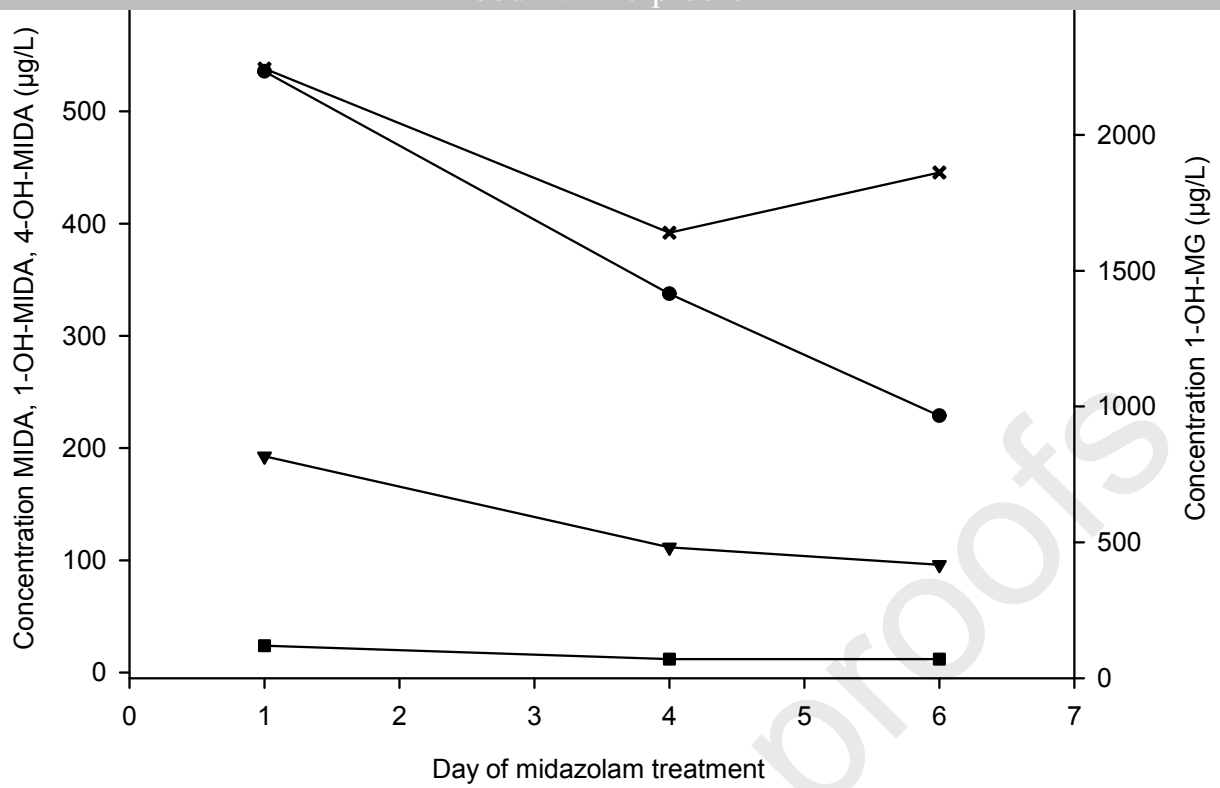


Figure 4: Concentrations of MIDA (●), 1-OH-MIDA (▼), 4-OH-MIDA (■) and 1-OH-MG (**x**) in a pediatric patient treated with MIDA.

MIDA and 1-OH-MG

Time (min)	Ammonium formate 20mM (%)	Methanol (%)
0.000	65.0	35.0
0.550	50.0	50.0
0.700	50.0	50.0
0.750	5.0	95.0
1.000	5.0	95.0
1.050	65.0	35.0
1.100	65.0	35.0

Table 2: Mass spectrometer settings for MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG and the internal standards

Compound	Precursor (m/z)	Product (m/z)	Collision energy (V)
Midazolam	326.2	291.1	27
$^{13}C_6$-Midazolam	332.2	297.1	27
1-Hydroxymidazolam	342.1	324.0	21
$^{13}C_6$-1-Hydroxymidazolam	348.1	330.0	21
4-Hydroxymidazolam	342.1	234.0	23
1-Hydroxymidazolam glucuronide	518.2	324.0	28
$^{13}C_6$-1-Hydroxymidazolam glucuronide	524.2	330.0	28

MIDA and 1-OH-MG QC samples in human serum

Compound	QC Level	Nominal concentration (µg/L)	Accuracy (%)	Precision (CV %)	
				Within-run (n=5)	Between-run (n=15)
MIDA	LLOQ	5	104.7	1.7	1.9
	LOW	25	96.9	0.6	0.3
	MEDIUM	500	98.5	0.5	0.4
	HIGH	1200	100.1	0.5	0.3
	DILUTION	500*	96.4	0.7	0.6
1-OH-MIDA	LLOQ	5	103.3	2.0	0.0
	LOW	25	95.9	0.8	0.3
	MEDIUM	500	97.0	0.5	0.2
	HIGH	1200	98.0	0.7	0.2
	DILUTION	500*	95.8	0.8	1.0
4-OH-MIDA	LLOQ	5	104.0	4.2	1.2
	LOW	25	97.6	2.1	0.7
	MEDIUM	500	103.5	0.8	0.8
	HIGH	1200	101.5	0.6	0.8
	DILUTION	500*	106.0	1.4	1.6
1-OH-MG	LLOQ	25	105.1	2.4	1.5
	LOW	125	94.9	1.2	1.3
	MEDIUM	1500	101.9	0.7	1.3
	HIGH	4000	104.1	0.6	0.3
	DILUTION	1500*	104.3	0.8	1.5

* Post-dilution (1:10)

serum

Compound	QC Level	Matrix factor	Matrix factor IS	IS normalized matrix factor (CV; n=5)	Recovery % (CV; n=5)
MIDA	LOW	1.023	1.053	0.971 (1.2%)	102.8 (1.3%)
	MEDIUM	1.019	1.057	0.964 (1.1%)	103.7 (3.3%)
	HIGH	1.036	1.023	1.012 (0.7%)	99.5 (2.2%)
1-OH-MIDA	LOW	1.010	1.036	0.975 (0.9%)	101.7 (1.7%)
	MEDIUM	1.008	1.046	0.964 (0.5%)	103.5 (2.7%)
	HIGH	1.016	1.014	1.002 (0.9%)	100.2 (2.6%)
4-OH-MIDA	LOW	1.042	1.036	1.005 (7.5%)	102.9 (5.3%)
	MEDIUM	0.990	1.046	0.946 (4.1%)	99.3 (5.0%)
	HIGH	0.993	1.014	0.979 (5.4%)	103.5 (3.6%)
1-OH-MG	LOW	1.126	1.144	0.984 (1.6%)	103.7 (1.7%)
	MEDIUM	1.037	1.049	0.989 (1.2%)	101.7 (2.4%)
	HIGH	1.063	1.061	1.002 (0.9%)	103.5 (3.6%)

temperature (accuracy values below 85% shown in bold)

Compound	Period	Matrix	QC Level	Accuracy (%)	Precision (CV%; n=5)
MIDA	7 days	serum	LOW	96.7	1.1
			MEDIUM	99.7	0.6
			HIGH	100.3	0.5
		EDTA plasma	LOW	99.2	1.0
			MEDIUM	100.4	0.3
			HIGH	100.7	0.4
		heparin plasma	LOW	100.1	0.9
			MEDIUM	101.3	0.2
			HIGH	100.6	0.4
		urine	LOW	95.4	0.5
			MEDIUM	101.2	0.7
			HIGH	101.0	0.4
1-OH-MIDA	7 days	serum	LOW	95.8	1.4
			MEDIUM	97.8	0.5
			HIGH	98.3	0.5
		EDTA plasma	LOW	98.1	2.0
			MEDIUM	100.2	0.2
			HIGH	100.5	0.6
		heparin plasma	LOW	100.5	1.2
			MEDIUM	101.8	0.2
			HIGH	100.9	0.6
		urine	LOW	95.2	0.8
			MEDIUM	101.8	0.4
			HIGH	101.0	0.5
4-OH-MIDA	7 days	serum	LOW	**82.8**	2.0
			MEDIUM	90.2	1.2
			HIGH	**78.7**	1.6
		EDTA plasma	LOW	86.1	1.1
			MEDIUM	88.6	0.8
			HIGH	87.3	0.4
		heparin plasma	LOW	**83.3**	0.7
			MEDIUM	**76.1**	1.6
			HIGH	**80.7**	0.4
		urine	LOW	**9.4**	0.0
			MEDIUM	**10.2**	1.4
			HIGH	**12.7**	1.1
4-OH-MIDA	3 days	serum	LOW	90.0	1.3
			MEDIUM	91.8	0.6
			HIGH	92.6	0.5
		EDTA plasma	LOW	91.1	0.4
			MEDIUM	92.4	0.9
			HIGH	91.6	0.8
		heparin plasma	LOW	89.6	1.9
			MEDIUM	90.6	0.7
			HIGH	86.3	0.8
		urine	LOW	**34.8**	11.2

			HIGH	**41.6**	0.9
4-OH-MIDA	24h	urine	LOW	**73.9**	14.0
			MEDIUM	**72.3**	1.7
			HIGH	**73.9**	1.1
1-OH-MG	7 days	serum	LOW	94.9	0.9
			MEDIUM	101.4	0.9
			HIGH	105.4	0.9
		EDTA plasma	LOW	100.6	0.6
			MEDIUM	101.2	0.4
			HIGH	101.9	0.3
		heparin plasma	LOW	100.7	0.9
			MEDIUM	100.4	0.6
			HIGH	100.9	0.3
		urine	LOW	100.1	0.7
			MEDIUM	99.7	1.0
			HIGH	100.5	0.3

Table 6: Stability of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG in human matrices stored in the refrigerator (accuracy values below 85% shown in bold)

Compound	Period	Matrix	QC Level	Accuracy (%)	Precision (CV%; n=5)
MIDA	7 days	serum	LOW	97.3	0.5
			MEDIUM	100.2	0.6
			HIGH	101.5	0.7
		EDTA plasma	LOW	98.4	0.3
			MEDIUM	99.4	0.2
			HIGH	99.6	0.6
		heparin plasma	LOW	99.1	0.3
			MEDIUM	100.4	0.2
			HIGH	99.3	0.7
		urine	LOW	96.3	0.6
			MEDIUM	101.4	0.7
			HIGH	101.5	0.9
1-OH-MIDA	7 days	serum	LOW	94.8	0.3
			MEDIUM	98.4	1.1
			HIGH	99.2	0.5
		EDTA plasma	LOW	96.6	1.2
			MEDIUM	99.2	0.2
			HIGH	99.2	0.6
		heparin plasma	LOW	99.0	0.3
			MEDIUM	100.8	0.3
			HIGH	99.8	0.6
		urine	LOW	95.8	0.3
			MEDIUM	101.6	0.6
			HIGH	100.7	0.5
4-OH-MIDA	7 days	serum	LOW	97.6	2.1
			MEDIUM	104.1	1.1
			HIGH	100.7	0.3
		EDTA plasma	LOW	97.4	0.9
			MEDIUM	100.5	1.5
			HIGH	100.7	0.8

			MEDIUM	100.5	0.9
			HIGH	100.0	0.7
		urine	LOW	**65.9**	2.2
			MEDIUM	**69.1**	1.2
			HIGH	**71.0**	0.9
4-OH-MIDA	3 days	urine	LOW	**81.3**	1.0
			MEDIUM	**81.4**	1.5
			HIGH	85.2	1.3
4-OH-MIDA	24h	urine	LOW	91.7	1.8
			MEDIUM	92.3	1.8
			HIGH	95.0	1.1
1-OH-MG	7 days	serum	LOW	94.1	1.0
			MEDIUM	100.9	0.7
			HIGH	104.0	0.7
		EDTA plasma	LOW	99.3	0.8
			MEDIUM	100.7	0.3
			HIGH	100.8	0.3
		heparin plasma	LOW	100.0	0.9
			MEDIUM	99.9	0.4
			HIGH	100.6	0.4
		urine	LOW	99.3	0.9
			MEDIUM	99.8	0.7
			HIGH	100.5	0.6

Table 7: Stability of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG in processed human matrices stored in the autosampler (at 10 °C)

Compound	Period	Matrix	QC Level	Accuracy (%)	Precision (CV%; n=5)
MIDA	7 days	serum	LOW	97.9	1.1
			MEDIUM	99.8	0.5
			HIGH	100.6	0.3
		EDTA plasma	LOW	100.1	0.8
			MEDIUM	100.6	0.3
			HIGH	101.0	0.3
		heparin plasma	LOW	100.4	0.8
			MEDIUM	101.4	0.9
			HIGH	101.2	0.4
		urine	LOW	100.9	0.8
			MEDIUM	100.9	0.6
			HIGH	100.6	0.9
1-OH-MIDA		serum	LOW	95.0	1.4
			MEDIUM	96.9	0.5
			HIGH	98.0	0.6
		EDTA plasma	LOW	99.2	0.7
			MEDIUM	99.7	0.6
			HIGH	99.5	0.4
		heparin plasma	LOW	100.2	0.5
			MEDIUM	99.6	0.7
			HIGH	99.3	0.1
		urine	LOW	100.7	0.8
			MEDIUM	99.9	0.6
			HIGH	101.7	0.8
4-OH-MIDA		serum	LOW	92.8	1.3

		HIGH	96.6	1.0
	EDTA plasma	LOW	99.7	1.0
		MEDIUM	98.5	0.5
		HIGH	98.2	0.4
	heparin plasma	LOW	97.2	1.2
		MEDIUM	96.4	0.2
		HIGH	95.0	0.9
	urine	LOW	99.4	1.5
		MEDIUM	101.6	1.0
		HIGH	99.7	0.4
1-OH-MG	serum	LOW	96.3	2.2
		MEDIUM	103.3	0.5
		HIGH	105.2	0.8
	EDTA plasma	LOW	105.7	2.8
		MEDIUM	100.9	0.5
		HIGH	101.4	0.7
	heparin plasma	LOW	100.3	1.3
		MEDIUM	101.5	0.6
		HIGH	100.3	0.2
	urine	LOW	104.4	1.2
		MEDIUM	100.7	0.6
		HIGH	101.3	0.7

Table 8: Stability of MIDA, 1-OH-MIDA, 4-OH-MIDA and 1-OH-MG in human matrices after three freeze-thaw cycles

Compound	Matrix	QC Level	Accuracy (%)	Precision (CV%; n=5)
MIDA	serum	LOW	99.0	0.7
		MEDIUM	100.1	0.8
		HIGH	100.8	0.8
	EDTA plasma	LOW	97.4	0.1
		MEDIUM	99.9	0.3
		HIGH	100.1	0.3
	heparin plasma	LOW	100.4	0.4
		MEDIUM	99.9	0.6
		HIGH	100.3	0.3
	urine	LOW	99.4	1.4
		MEDIUM	100.0	0.8
		HIGH	97.0	1.3
1-OH-MIDA	serum	LOW	98.2	1.2
		MEDIUM	98.2	0.5
		HIGH	99.1	0.3
	EDTA plasma	LOW	95.7	0.5
		MEDIUM	100.5	0.2
		HIGH	99.8	0.6
	heparin plasma	LOW	100.1	0.6
		MEDIUM	100.0	0.6
		HIGH	100.4	0.8
	urine	LOW	100.5	1.0
		MEDIUM	99.8	0.6
		HIGH	97.2	1.2
4-OH-MIDA	serum	LOW	94.5	1.6
		MEDIUM	101.7	0.8

	EDTA plasma	LOW	98.5	1.3
		MEDIUM	98.8	0.8
		HIGH	98.0	0.9
	heparin plasma	LOW	96.1	2.0
		MEDIUM	98.7	1.7
		HIGH	97.6	1.3
	urine	LOW	89.3	1.5
		MEDIUM	91.6	2.3
		HIGH	90.8	1.8
1-OH-MG	serum	LOW	94.6	3.5
		MEDIUM	101.4	0.5
		HIGH	105.4	0.5
	EDTA plasma	LOW	98.5	1.1
		MEDIUM	100.5	0.9
		HIGH	100.1	0.6
	heparin plasma	LOW	97.1	1.6
		MEDIUM	100.4	0.4
		HIGH	99.8	0.6
	urine	LOW	98.9	0.8
		MEDIUM	100.6	0.3
		HIGH	98.3	1.3

A.M.A. Wessels: Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft

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W. Bult: Writing - Review & Editing

M.W.N. Nijsten: Resources, Writing - Review & Editing

M.C.J. Kneijber: Resources, Writing - Review & Editing

D.J. Touw: Conceptualization, Resources, Writing - Review & Editing, Supervision

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

- The runtime of the assay for midazolam and its metabolites is only 1.1 minute.
- No deglucuronidation step is needed to quantify 1-hydroxymidazolam glucuronide.
- The validated assay is applicable to quantification in serum, plasma and urine.
- The UHPLC-MS/MS method is suitable for routine analysis and for large studies.